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Taxonomy and ecology of *Botryosphaeria* species and their anamorphs from Venezuela

by

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Declaration

I, the undersigned hereby declare that the thesis submitted herewith for the degree *Philosophiae Doctor* to the University of Pretoria, contains my own independent work, and has hitherto not been submitted for any degree at any other University.

Sari Ramón Mohali Castillo

April, 2006

Dedicated to my beloved wife, Enid, my sons Jesus and Andres, my parents Sari and Gladys and to my brothers and sister.

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PREFACE

The fungal genus *Botryosphaeria* including its anamorphs has a cosmopolitan distribution and occurs on a wide range of monocotyledonous, dicotyledonous and gymnospermous hosts, including woody twigs and branches, herbaceous leaves, stems of grasses, and even lichen thalli. These fungi give rise to a wide variety of symptoms such as shoot blights, stem cankers, fruit rots, die-back and gummosis. They are also known as saprophytes on dead or dying stems, branches or leaves of plants. In Venezuela, the following species have been reported: *Lasiodiplodia theobromae* (the anamorph of *Botryosphaeria rhodina*), *Diplodia pinea*, *D. mutila* and *Dothiorella* spp. However, their characterization has been based only on morphological descriptions. The most common and well characterised species, *L. theobromae*, is associated with pines and other hosts.

The focus of the studies presented in this thesis as to survey *Eucalyptus* and *Acacia* plantations in Venezuela for the presence and influence of *Botryosphaeria* spp., and to characterise these fungi using morphological characteristics and DNA sequence data. I also evaluated the pathogenicity and population biology of *Botryosphaeria* species present in the plantations. It was anticipated that the various studies would characterize a relatively large number of *Botryosphaeria* species, potentially recognise new species and provide some perspective of their relative importance to the *Eucalyptus* growing industry.

The thesis has been divided into chapters that reflect discrete units prepared for future publication. The first chapter presents a review of the relevant literature. Two chapters treat the taxonomy of the *Botryosphaeria* spp. collected in this study. An additional two chapters consider the population biology of the most commonly

encountered species and the remaining chapter deals with the pathogenicity of these fungi to *Eucalyptus*.

In the literature review I treat the taxonomy of *Botryosphaeria*, which has been the subject of much uncertainty for many years. I also briefly review recent findings related to their molecular characterization. A focus is also placed on the pathogenicity of *Botryosphaeria* species on various host plants and their known importance in Venezuela. The focus concerns mainly *Eucalyptus* spp.

Surveys that formed part of this study logically gave rise to a large collection of *Botryosphaeria* isolates. An important component of this thesis was to characterise these fungi. This was done based on morphological characteristics and also comparisons of DNA sequence data for various gene regions known to be informative for these fungi.

Lasiodiplodia theobromae (anamorph of *B. rhodina*), *B. ribis* and *B. parva* were studied from a population biology perspective. These fungi were chosen for study because they were the most common species encountered on *Eucalyptus* in Venezuela. To study populations, I made use of simple sequence repeat markers (SSR). Three populations of each of *L. theobromae* (Venezuela, Mexico and South Africa) and *B. ribis-B. parva* complex (Venezuela, Colombia and Hawaii) were analysed and the data considered in terms of population differentiation, gene flow, mode of reproduction, gene and genotype diversity.

In the final chapter of this thesis, I evaluated the pathogenicity of the seven *Botryosphaeria* species identified from Venezuela. These seven species were thus inoculated on *Eucalyptus urophylla* x *E. grandis* hybrid clones. A second series of inoculations was then carried out with the two most pathogenic fungi to determine the relative tolerance of the most important clones to infection.

This thesis was conducted over a period of four years. Work was undertaken both in Venezuela and South Africa and this necessitated long periods of time away from my home University and family. Surveys were conducted in many parts of Venezuela to collect the *Botryosphaeria* spp. of interest for latter study in South Africa. The research chapters have been completed systematically and they evolved over time. Each represents a discrete unit implying that there is some overlap, at least in the references, between them. I would like to believe that the thesis will provide a firm foundation for further studies of *Botryosphaeria* spp. and prove valuable to the small but important *Eucalyptus* growing industry in Venezuela.

SUMMARY

Species in the fungal genus *Botryosphaeria* are generally regarded as weak pathogens that attack stressed or wounded plants. Such stresses include drought, hail, wind and frost damage or insect infestation. It is interesting in this regard that these fungi have, in recent years, also been shown to exist endophytically in asymptomatic tissue as latent pathogens on *Eucalyptus*.

The taxonomy of *Botryosphaeria* spp. has been problematic from more than 100 years. The identification of species in this genus has been traditionally based on morphological descriptions, which tend to be interpreted subjectively. In recent years, molecular techniques and particularly analyses of DNA sequences have been used to identify closely related *Botryosphaeria* species. Thus, in the first chapter of this thesis, a combination of morphological characteristics and analyses of multiple gene sequences were successfully used to identify cryptic *Botryosphaeria* species occurring in Venezuela.

Studies in this thesis have treated the taxonomy, phylogenetic relationships, population structure and pathogenicity of *Botryosphaeria* species. The species were isolated in various areas of Venezuela and from various woody hosts, such as *Acacia*, *Eucalyptus* and *Pinus*. The DNA based comparisons used to identify *Botryosphaeria* spp. were based on sequence data for the ITS regions of the ribosomal DNA operon. These sequences were from fragments including the 3' end of the small subunit (SSU) rRNA gene, the internal transcribed spacer ITS (ITS1), the complete 5.8S rRNA gene, the ITS2 and the 5' end of the large subunit (LSU) rRNA gene. In addition, part of the EF 1-α was amplified using the primers EF1-728F and EF1-986R. In total, seven *Botryosphaeria* spp. were characterised from Venezuela. Two new *Botryosphaeria*

anamorphs, *Fusicoccum andinum prov. nom.* and *F. stromaticum prov. nom.* (chapter 2), were identified using the combination of the two-gene regions (ITS and EF1-α). An additional five *Botryosphaeria* species, *Botryosphaeria mamane*, *B. dothidea*, *B. rhodina*, *B. parva* and *B. ribis*, were characterized using the ITS region (chapter 3).

The population biology for *L. theobromae* and the *B. ribis-B. parva* complex (Chapter 4 and 5) was considered using SSR markers previously developed for these fungi. Three populations of *L. theobromae* (Venezuela, Mexico and South Africa) and *B. ribis-B. parva* complex (Venezuela, Colombia and Hawaii) were used in each of the comparisons. *Lasiodiplodia theobromae* showed no evidence for host specificity, and there was very high gene flow between populations of isolates from different hosts. Reproduction was predominantly clonal with some genotypes widely distributed within a region. The isolates representing the *B. ribis* - *B. parva* complex were separated into two distinct groups, confirming the species barrier within this species complex that has been reported previously based on combined gene genealogies, and SSR and PCR-RFLP data from other populations. The *B. ribis* populations had a low degree of isolation and an asexual (clonal) mode of reproduction. Populations of *B. parva* in Colombia and Hawaii showed recombination within each population, and a high degree of isolation between Colombia and Hawaii.

Pathogenicity tests were undertaken on *Eucalyptus urophylla* x *E. grandis* hybrid clones in the field using the seven *Botryosphaeria* species identified in an earlier study (Chapter 3). These tests showed that not all *Botryosphaeria* species collected from *Eucalyptus*, *Acacia* and *Pinus* in Venezuela are pathogenic on the selected *Eucalyptus*-hybrid. *Botryosphaeria parva* and *B. ribis* were the most pathogenic on *Eucalyptus*, but of the five clones inoculated, one had obviously greater tolerance to infection by these species. The remainder of the clones inoculated were less tolerant to infection.

Botryosphaeria parva gave rise to longer lesions than did isolates of *B. ribis* and the former species appears to be most pathogenic.

This study represents the first comprehensive treatment of *Botryosphaeria* spp. in Venezuela. The focus has been on species from forestry crops, but it is clear that these fungi are well represented in the country and I believe that many new species remain to be discovered. It is my hope that studies presented in this thesis will not only provide a foundation for future work on *Botryopshaeria* spp., but that they will also stimulate an elevated interest in the field of forest pathology.

OPSOMMING

Spesies in die fungus genus *Botryosphaeria* word as swak patogene beskou wat slegs plante onder stres of gewonde plante aanval. Sodanige stres op plante word veroorsaak deur droogte, hael, wind- en rypskade of insekinfestasie. Dit is ook onlangs getoon dat die swamme endofities as latente patogene in gesonde plantweefsel kan leef.

Die taksonomie van *Botryosphaeria* spp. is al vir meer as 'n 100 jaar 'n probleem. Die identifikasie van spesies in die genus is tradisioneel op morfologiese beskrywings gebaseer, wat dikwels ruimte laat vir subjektiewe interpretasies. Molekulêre tegnieke, in besonder analises van DNA basisopeenvolging, is onlangs gebruik om nabyverwante *Botryosphaeria* spp. te onderskei. Vir hierdie rede word 'n kombinasie van morfologiese karakters en DNA basisopeenvolging data van verskillende gene in Hoofstuk 1 gebruik om kriptiese *Botryosphaeria* spp. in Venezuela te identifiseer.

Die verskillende studies in hierdie tesis ondersoek die taksonomie, filogenetiese verwantskappe, populasie struktuur en patogenisiteit van *Botryosphaeria* spp. in verskillende areas van Venezuela en van verskillende gashere soos *Acacia*, *Eucalyptus* en *Pinus*. Die DNA gebaseerde vergelykings tussen verskillende *Botryosphaeria* spp. is eerstens geskoei op DNA basisopeenvolging van die ITS gedeelte van die ribosomale DNA operon, insluitende die 3' kant van die SSU rRNA, die hele ITS1, 5.8S geen en ITS2, asook die 5' kant van die LSU rRNA geen. Hierbenewens is gedeeltes van die EF1-α geen geamplifiseer met behulp van die primers EF1-728F en EF1-986R. Sewe *Botryosphaeria* spp. is met behulp van die data in Venezuela geïdentifiseer. Twee nuwe *Botryosphaeria* anamorwe, *Fusicoccum andinum prov. nom.* en *F. stromaticum prov. nom.* (Hoofstuk 2) is geïdentifiseer met behulp van die twee geen areas (ITS en EF1- α).

'n Addisionele vyf *Botryosphaeria* spesies, *B. mamane*, *B. dothidea*, *B. rhodina*, *B. parva* en *B. ribis* is geidentifiseer met behulp van ITS data en morfologie (Hoofstuk 3).

Die populasie biologie van *Lasiodiplodia theobromae* en die *B. ribis-B. parva* kompleks (Hoofstukke 4 en 5) was ondersoek met behulp van SSR merkers wat vroeër vir die swamme ontwikkel is. Drie populasies elk van *L. theobromae* (Venezuela, Mexico en Suid-Afrika) en die *B. ribis – B parva* kompleks (Venezuela, Colombia en Hawaii) is ondersoek. Daar was geen aanduiding van gasheer seleksie in populasies van *L. theobromae*, met hoë vlakke van geenbeweging tussen die populasies van verskillende gashere. Reproduksie was hoofsaaklik klonaal, met sommige genotipes wydverspreid in die areas. Die isolate wat die *B. ribis* - *B. parva* kompleks verteenwoordig het, is in twee groepe verdeel gebaseer op die SSR data. Hierdie data bevestig die hipotese van 'n spesiegrens tussen *B. parva* en *B. ribis* soos vroeër gevind uit 'n studie van multigeen DNA basisopeenvolging, en SSR en PCR-RFLP data van ander populasies. Die *B. ribis* populasies was swak onderskei (hoë geenvloei) en hoofsaaklik aseksuele reproduksie. Populasies van *B. parva* vanaf Hawaii and Colombia was egter afgeskei (lae geenvloei) en toon beide tekens van seksuele reproduksie.

Patogenisiteitsproewe is gedoen met die sewe *Botryosphaeria* spp., wat in Hoofstuk 3 geïdentifiseer is, op *Eucalyptus urophylla* x *E. grandis* hibried klone in die veld. Hierdie proewe het gewys dat nie al die *Botryosphaeria* spp. patogenies is op die *Eucalyptus* hybried nie. *Botryosphaeria parva* en *B. ribis* was die mees patogeniese spesies op *Eucalyptus*, maar van die vyg klone wat getoets was het ten minste een beduidende weerstand teen die patogene gehad. Die oorblywende klone was minder bestand teen infeksie. *Botryosphaeria parva* het langer letsels veroorsaak as *B. ribis*, en die spesie blyk dus die mees patogeniese te wees.

Hierdie studie verteenwoordig die eerste oorhoofse ondersoek na *Botryosphaeria* spp. in Venezuela. Die fokus was op spesies geassosieer met bosboubome, maar dit is duidelik dat die swamme goed verteenwoordig is in die land en daar is waarskynlik meer onontdekte spesies. Dit is my hoop dat die studies wat hier verteenwoordig word nie net die fondasie sal vorm van toekomstige werk op *Botryosphaeria* nie, maar dat dit ook navorsing in die veld van bosboupatologie as geheel sal stimuleer.